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Sorafenib suppresses growth and survival of hepatoma cells by accelerating degradation of enhancer of zeste homolog 2

Shanshan Wang,[1] Yu Zhu,[2] Hongyong He,[3] Jing Liu,[4] Le Xu,[2] Heng Zhang,[3] Haiou Liu,[1] Weisi Liu,[1] Yidong Liu,[1] Deng Pan,[1] Lin Chen,[1] Qian Wu,[1] Jiejie Xu[1,5] and Jianxin Gu[1]

[1]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences; Departments of [2]Urology; [3]General Surgery, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai; [4]School of Medicine, Nantong University, Nantong, China

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Enhancer of zeste homolog 2 (EZH2) is a mammalian histone methyltransferase that contributes to the epigenetic silencing of target genes that regulate cancer cell growth and survival. It is overexpressed in hepatocellular carcinoma (HCC) with a clinical significance that remains obscure. Sorafenib, a multikinase inhibitor, has been used as a first-line therapeutic drug and shown clinical efficiency for advanced-stage HCC patients. In the present study, we found that sorafenib lowered the protein level of EZH2 through accelerating proteasome-mediated EZH2 degradation in hepatoma cells. Overexpression of EZH2 reversed sorafenib-induced cell growth arrest, cell cycle arrest, and cell apoptosis dependent on histone methyltransferase activity in hepatoma cells. More importantly, shRNA-mediated EZH2 knockdown or EZH2 inhibition with 3-deazaneplanocin A treatment promoted sorafenib-induced hepatoma cell growth arrest and apoptosis. Sorafenib altered the hepatoma epigenome by reducing EZH2 and H3K27 trimethylation. These results revealed a novel therapeutic mechanism underlying sorafenib treatment in suppressing hepatoma growth and survival by accelerating EZH2 degradation. Genetic deletion or pharmacological ablation of EZH2 made hepatoma cells more sensitive to sorafenib, which helps provide a strong framework for exploring innovative combined therapies for advanced-stage HCC patients. (*Cancer Sci* 2013; 104: 750–759)

Primary liver cancer, predominantly hepatocellular carcinoma (HCC), is a major public health problem worldwide and the third most common cause of cancer-related mortality globally.[1] One of the main reasons for the high mortality rate in patients with HCC is the lack of effective therapeutic options, especially for those with advanced disease.[2] Potentially curative therapies like surgical resection, transplantation, and percutaneous ablation are only available to limited patients with early HCC.[3] Moreover, the majority of patients with HCC present with advanced disease due to the asymptomatic nature of early HCC, lack of awareness, and poorly defined clinical screening strategies.[4] The paucity of effective and well-tolerated treatments for advanced HCC highlights the desperate need for new therapeutic approaches for this deadly disease.

The absence of standard systemic therapy for patients with advanced HCC has changed with the recent positive randomized controlled trial testing sorafenib, which represents a breakthrough in the management of this neoplasm.[5] The unprecedented results of a recently published phase III clinical trial show that sorafenib significantly improves survival and time to progression in patients with advanced HCC.[5] Unfortunately, less than half of patients with advanced-stage HCC benefit from these therapies, and the benefits are transient resulting from quickly acquired adaptive resistance to sorafenib treatment.[6] These benefits and challenges highlight the importance of underlying molecular mechanism exploration for sorafenib treatment and novel oncogenic mechanism in this malignancy. Molecular combination therapies are currently being tested that block the main pathways involved in hepatocarcinogenesis, such as mammalian target of rapamycin, c-MET, insulin-like growth factor, and fibroblast growth factor signaling, among others.[7] Increasing evidence reveals that epigenetic modification can be viewed as "on" and "off" switches for gene expression, where shutting down tumor-suppressor genes or activating oncogenes can lead to aberrant cellular proliferation and apoptosis,[8] indicating epigenetic modulators might be used as a new weapon in this combination war against advanced-stage HCC.

Enhancer of zeste homolog-2 (EZH2), the catalytic subunit of polycomb repressive complex 2 (PRC2), has been identified as the crucial methyltransferase that catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3) to mediate epigenetic transcriptional silencing of target gene expression.[9] This function of EZH2 plays a key role in controlling biological processes including X-chromosome inactivation, germline development, stem cell pluripotency maintenance, and tumor development.[10,11] Overexpression of EZH2 has been implicated in tumorigenesis of malignancies derived from prostate, breast, skin, bladder, stomach, liver, and other organs.[12–17] Given the crucial putative oncogenic function of EZH2 in hepatocarcinogenesis,[18–25] epigenetic modulators directing EZH2, such as 3-deazaneplanocin A (DZNep), might be used as promising therapeutic agents against liver cancer.[26,27] However, little is known about the potential significance of epigenome alterations mediated by EZH2 in liver cancer after sorafenib treatment.

In this study, we showed that sorafenib could decrease the EZH2 protein level by accelerating proteasome-mediated EZH2 degradation in hepatoma cells. Furthermore, hepatoma cells overexpressing EZH2 with histone methyltransferase (HMT) activity had enhanced resistance to sorafenib-induced cell growth arrest, cell cycle arrest, and apoptosis, whereas hepatoma cells with depletion of EZH2 activity using shRNA transfection or DZNep treatment were relatively more sensitive to sorafenib-induced cell growth arrest or apoptosis. Sorafenib altered the hepatoma epigenome by reducing the EZH2-mediated H3K27me3 of target genes. These data reveal the potential significance of EZH2-mediated hepatoma epigenomic alterations in sorafenib treatment, indicating that a new combination therapy with sorafenib and EZH2 inhibitor DZNep is an

[5]To whom correspondence should be addressed.
E-mail: jjxufdu@fudan.edu.cn

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attractive strategy for controlling tumor progression, especially in advanced-stage HCC patients with high levels of EZH2.

Materials and Methods

Cell culture. Human hepatoma cell lines HepG2, 7404, Huh7, and SK-Hep1 were obtained directly from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. The cell lines have been characterized at the cell bank by DNA fingerprinting analysis using short tandem repeat markers. All cell lines were placed under cryostage after they were obtained from the cell bank and used within 6 months of thawing fresh vials, as described previously.[28]

Construction of plasmids. Expression plasmids encoding wild-type EZH2 and HMT deletion mutant EZH2-ΔSET were constructed as described previously.[17] Luciferase reporter plasmid driven by the 5′-flanking region of EZH2 (−1876 upstream to +3) were amplified from human genome DNA of HepG2 cells and constructed in pGL3-Basic vector as described previously.[29] The PCR primer sets used are shown in Table S1. All plasmid constructs were confirmed by DNA sequencing.

Plasmid transfection and RNAi. Transient and stable transfections with various plasmids were carried out as previously described.[30] The shRNA against *EZH2* gene EZH2 shRNA and corresponding control shRNA (Sigma, St. Louis, MO, USA) were used for RNA interference as described previously.[31] Gene silencing effects were confirmed by Western blot analysis and RT-PCR at 48 h post-transfection.

Western blot. Western blot analysis was carried out as previously described.[32,33] Primary antibodies included those against EZH2, cleaved poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Danvers, MA, USA), GAPDH, HA tag (Santa Cruz Biotechnology, Santa Cruz, CA, USA), H3K27me3 (Millipore, Billerica, MA, USA), H3 (Abcam, Cambridge, MA, USA), and proliferating cell nuclear antigen (PCNA; BD Biosciences, Rockville, MD, USA).

Quantitative RT-PCR. Total RNA extraction from cultured cells and quantitative RT-PCR (qRT-PCR) analyses were carried out as described previously.[30] The PCR primer sets used are shown in Table S2.

Luciferase activity assay. The pGL3-EZH2-Pro plasmid containing EZH2 promoter and pGL3-Basic control vector were used for assessing the effect of sorafenib treatment on EZH2 transcriptional activity. Luciferase activity assay was carried out as described previously.[28]

5-Bromodeoxyuridine incorporation assay, cell proliferation assay, colony formation assay, and cell cycle analysis. The BrdU incorporation assay, cell proliferation assay, colony formation assay, and cell cycle analysis were carried out as described preiously.[28,30,31] The procedures are described in detail in Data S1.

Annexin V/propidium iodide staining, TUNEL assay, and caspase 3/7 activity assay. Annexin V/propidium iodide (PI) staining, TUNEL assay, and caspase 3/7 activity assay were carried out using the Annexin V–FITC Apoptosis Detection Kit (BD Biosciences), In Situ Cell Death Detection Kit, AP (Roche Applied Science, Mannheim, Germany), and Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturers' instructions.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was carried out with the ChIP Assay Kit (Millipore) according to the manufacturer's instructions. The percentage of the bound DNA was quantified against the original DNA input by PCR analysis. The PCR primer sets used are shown in Table S3.

Statistical analysis. Data are presented as the mean ± SEM of at least three independent replicates using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and comparisons between different groups assessed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Sorafenib decreases EZH2 protein levels through accelerating proteasome-mediated degradation in hepatoma cells. To explore the putative significance of EZH2-mediated epigenetic alterations in molecular targeted therapy with sorafenib treatment in HCC, we investigated the protein levels of EZH2 and H3K27me3 to assess the alterations of EZH2 and its HMT activity in hepatoma cells after sorafenib treatment. We found a marked decrease in EZH2 protein levels and associated global levels of H3K27me3 histone mark in HepG2, 7404, Huh7, and SK-Hep1 cells after sorafenib treatment (Fig. 1a). Moreover, we also found sorafenib downregulated EZH2 protein levels and global H3K27me3 levels in a dose- and time-dependent manner (Fig. 1b,c). However, sorafenib treatment had no significant effect on EZH2 mRNA levels as quantified by qRT-PCR analysis in HepG2, 7404, Huh7, or SK-Hep1 cells (Fig. 1d). Furthermore, luciferase activity assay using EZH2 promoter showed that no significant alterations were found in Huh7 cells after sorafenib treatment (Fig. 1e). These data indicate that sorafenib downregulates EZH2 and its HMT activity in hepatoma cells in a transcription-independent manner.

To dissect the molecular mechanisms underlying the downregulation of EZH2 protein levels in hepatoma cells after sorafenib treatment, we evaluated the potential alterations of EZH2 protein stability through inhibiting *de novo* protein synthesis with cycloheximide (CHX) treatment and inhibiting proteasome-mediated protein degradation with MG132. We found more rapid downregulation of EZH2 protein stability in 7404 and Huh7 cells after combined CHX and sorafenib treatment compared to CHX treatment alone, indicating downregulation of EZH2 protein levels might be due to destabilized EZH2 protein after sorafenib treatment in hepatoma cells (Fig. 2a). We also found that decreased EZH2 protein levels in 7404 and Huh7 cells after sorafenib treatment were reversed by proteasome inhibitor MG132, suggesting that the decline in EZH2 protein levels could be attributable to accelerating proteasome-mediated EZH2 protein degradation in hepatoma cells after sorafenib treatment (Fig. 2b). Taken together, these results indicate that sorafenib accelerated proteasome-mediated EZH2 protein degradation, thus declining EZH2 protein levels and associated HMT activities in hepatoma cells.

Hepatoma cells' resistance to sorafenib is dependent EZH2 HMT activity. To address the clinical significance of EZH2 protein levels and associated decline in HMT activities mediated by sorafenib treatment, we analyzed functional alterations after sorafenib treatment in 7404 and Huh7 cells with overexpression of wild-type EZH2 and HMT deletion mutant EZH2-ΔSET. We found that the markedly decreased proliferative marker PCNA in 7404 and Huh7 cells treated with sorafenib was rescued by wild-type EZH2 overexpression, but not EZH2-ΔSET transfection, indicating the crucial role of declined EZH2 protein levels and associated HMT activities in sorafenib-blunted proliferation in hepatoma cells (Fig. 3a). Moreover, the BrdU incorporation assay (Fig. 3b) and cell proliferation assay (Fig. 3c) further confirmed the pivotal role of EZH2 and its HMT activity in 7404 and Huh7 cells after sorafenib treatment. Colony formation assay showed that wild-type EZH2 overexpression, but not EZH2-ΔSET transfection, rescued the decline in anchorage-independent growth in 7404 and Huh7 cells treated with sorafenib (Fig. 3d). In addition, flow cytometry analysis of cell cycle revealed that significant G_0/G_1 cell cycle arrest in 7404 and Huh7 cells treated with

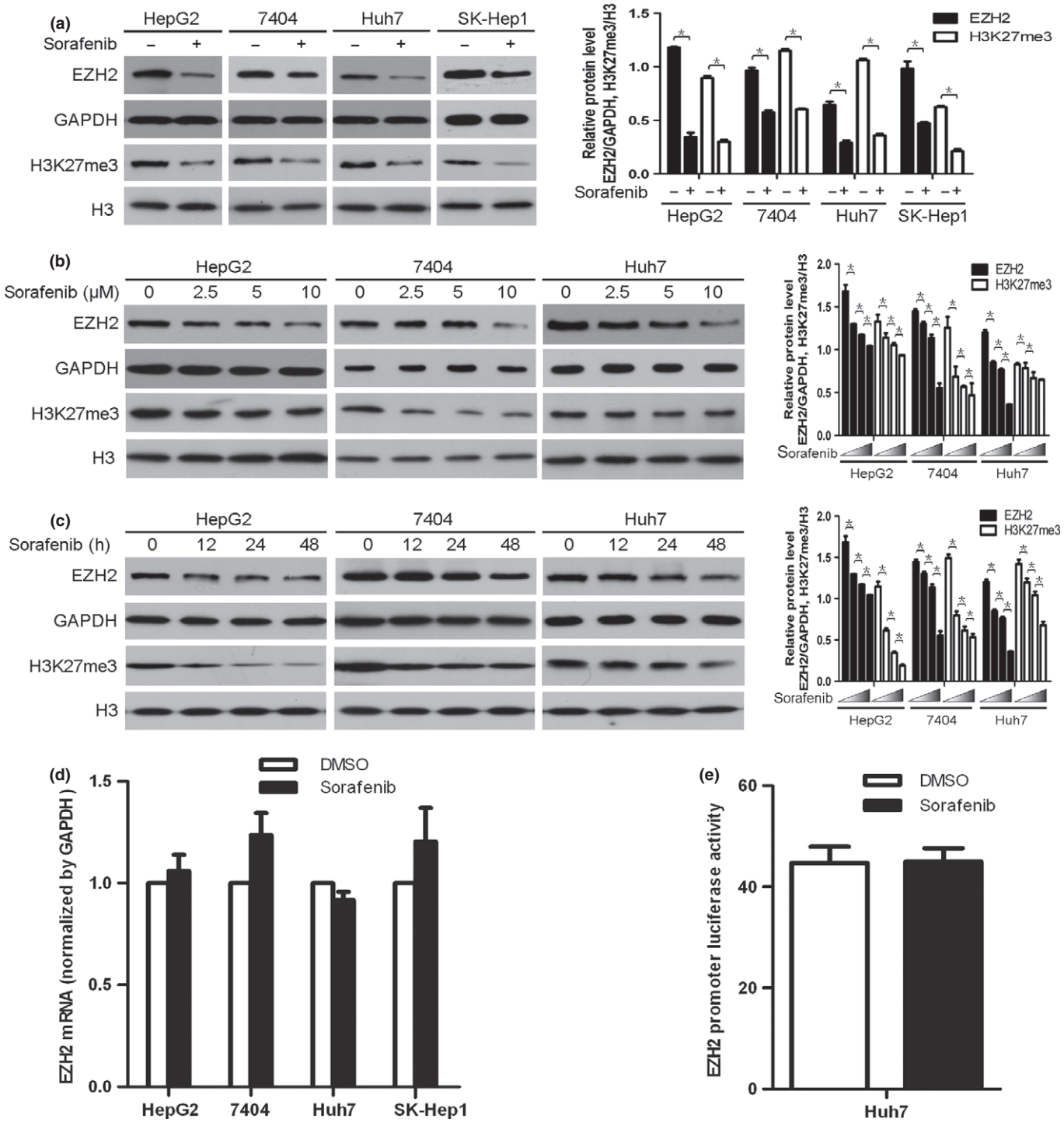

Fig. 1. Sorafenib lowers enhancer of zeste homolog 2 (EZH2) protein levels in hepatoma cells. (a) Western blot analysis of EZH2, GAPDH, trimethylation of lysine 27 of histone H3 (H3K27me3), and total H3 protein levels in HepG2, 7404, Huh7, and SK-Hep1 cells after sorafenib (5 μM) treatment for 48 h. (b) Western blot analysis of EZH2, GAPDH, H3K27me3, and total H3 protein levels in HepG2, 7404, and Huh7 cells after sorafenib (0, 2.5, 5, and 10 μM) treatment for 24 h. (c) Western blot analysis of EZH2, GAPDH, H3K27me3, and total H3 protein levels in HepG2, 7404, Huh7, and SK-Hep1 cells after sorafenib (5 μM) treatment for the indicated times. (d) Quantitative RT-PCR analysis of EZH2 mRNA levels in HepG2, 7404, Huh7, and SK-Hep1 cells after sorafenib (5 μM) treatment for 48 h. (e) Luciferase activity analysis of EZH2 promoter transcriptional activity in Huh7 cells after sorafenib (5 μM) treatment for 48 h. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a *t*-test (*$P < 0.05$).

sorafenib was rescued by wild-type EZH2 overexpression, but not EZH2-ΔSET transfection (Fig. 3e).

Furthermore, Annexin V/PI staining (Fig. 4a), TUNEL assay (Fig. 4b), caspase 3/7 activity assay (Fig. 4c), and Western blot analysis of cleaved PARP (Fig. 4d) revealed that significantly increased cell apoptosis in 7404 and Huh7 cells treated with sorafenib was reversed by wild-type EZH2 overexpression, but not EZH2-ΔSET transfection, suggesting that

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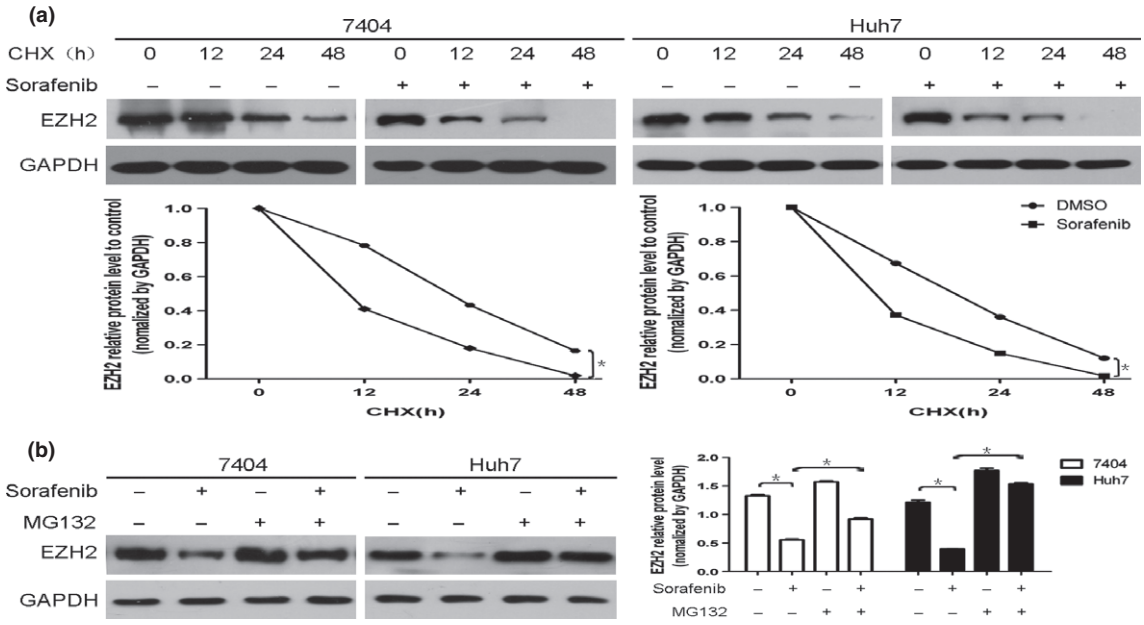

Fig. 2. Sorafenib accelerates proteasome-dependent enhancer of zeste homolog 2 (EZH2) protein degradation. (a) Western blot analysis of EZH2 and GAPDH protein levels in 7404 and Huh7 hepatoma cells after cycloheximide (CHX; 10 μg/mL) treatment without or with sorafenib (5 μM) for the indicated times. (b) Western blot analysis of EZH2 and GAPDH protein levels in 7404 and Huh7 cells after sorafenib (5 μM for 48 h) treatment without or with MG132 (10 μM for 6 h). The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a *t*-test (*$P < 0.05$).

overexpression of EZH2 blocks sorafenib-induced cell apoptosis dependent on HMT activity. Taken together, these data elucidate the crucial significance of declined EZH2 protein levels and associated HMT activities in functional alterations, including proliferation inhibition, anchorage-independent growth declination, cell cycle arrest, and apoptosis, after sorafenib treatment in hepatoma cells.

Knockdown and inhibition of EZH2 sensitizes hepatoma cells to sorafenib treatment. To determine the potential impact of EZH2 inhibition on molecular targeted therapy against advanced-stage HCC with sorafenib, we assessed functional alterations in the presence of shRNA-mediated EZH2 knockdown or DZNep-induced EZH2 inhibition in hepatoma cells after sorafenib treatment. Treatment with EZH2 inhibitor DZNep or cotransfection with EZH2-specific shRNA further facilitated the decrease in EZH2 protein levels and associated decline in HMT activity in 7404 and Huh7 cells after sorafenib treatment (Fig. 5a). More importantly, cell proliferation assay showed the synergetic effect of DZNep-mediated EZH2 inhibition and shRNA-mediated EZH2 knockdown on cell proliferation declination in 7404 and Huh7 cells treated with sorafenib (Fig. 5b). Colony formation assay confirmed DZNep-mediated EZH2 inhibition and shRNA-mediated EZH2 knockdown synergized sorafenib-induced inhibition of anchorage-independent growth in 7404 and Huh7 cells (Fig. 5c). The TUNEL assay (Fig. 5d) and Annexin V/PI staining (Fig. 5e) further indicated that DZNep-mediated EZH2 inhibition and shRNA-mediated EZH2 knockdown strengthened sorafenib-mediated cell apoptosis in 7404 and Huh7 cells. Overall, these data indicated that DZNep-mediated EZH2 inhibition and shRNA-mediated EZH2 knockdown sensitized hepatoma cells to sorafenib treatment.

Sorafenib induces EZH2-mediated cancer epigenomic alterations in hepatoma cells. Given the putative significance of EZH2 declination in combined molecular targeted therapies against HCC, we further investigated EZH2-mediated cancer epigenomic alterations on PRC2 target genes in hepatoma cells after sorafenib treatment. First, qRT-PCR analysis showed remarkable upregulated mRNA levels of PRC2 target genes such as *ADRB2*, *DAB2IP*, *Runx3*, *CIITA*, *CDH1*, *p16*, and *p21* in sorafenib-treated 7404 and Huh7 cells in a dose-dependent manner (Fig. 6a). Moreover, qRT-PCR analysis revealed that mRNA levels of these genes could be decreased by wild-type EZH2 overexpression, but increased by EZH2-ΔSET transfection or EZH2-specific shRNA infection, in 7404 and Huh7 cells (Fig. 6b). In addition, overexpression of wild-type EZH2, but not EZH2-ΔSET, rescued upregulated mRNA levels of the PRC2 target genes in sorafenib-treated 7404 and Huh7 cells (Fig. 6c). Furthermore, ChIP assay showed that significant reductions of the recruitment of EZH2 and the occupancy of the H3K27me3 histone mark at the promoter of known PRC2 target genes such as *ADRB2*, *DAB2IP*, *CDH1*, and *p16* in sorafenib-treated 7404 and Huh7 cells (Fig. 6d). These data indicated that sorafenib regulated the cancer epigenome by reducing the recruitment of EZH2 and the occupancy of the H3K27 histone mark in hepatoma cells.

Discussion

The positive results obtained by sorafenib in advanced-stage HCC represent a breakthrough in the understanding of molecular mechanisms underlying this deadly malignancy, a landmark advancement in improving survival for patients with advanced HCC, and the proof-of-principle that molecular targeted therapies have a curative role in this otherwise treatment-resistant disease.[34] However, the molecular complexity of HCC warrants further efforts in the direction of combining therapies targeting enriched molecules to approach a more personalized treatment of this neoplasm. Our study identified proteasome-mediated EZH2 protein degradation and subsequent cancer epigenomic alterations dependent on HMT activity as crucial molecular events for hepatoma cell growth inhibition and apoptosis undergoing sorafenib treatment, and revealed that EZH2 knockdown and inhibition sensitizes hepatoma cells to sorafenib treatment. These data elucidate a novel molecular

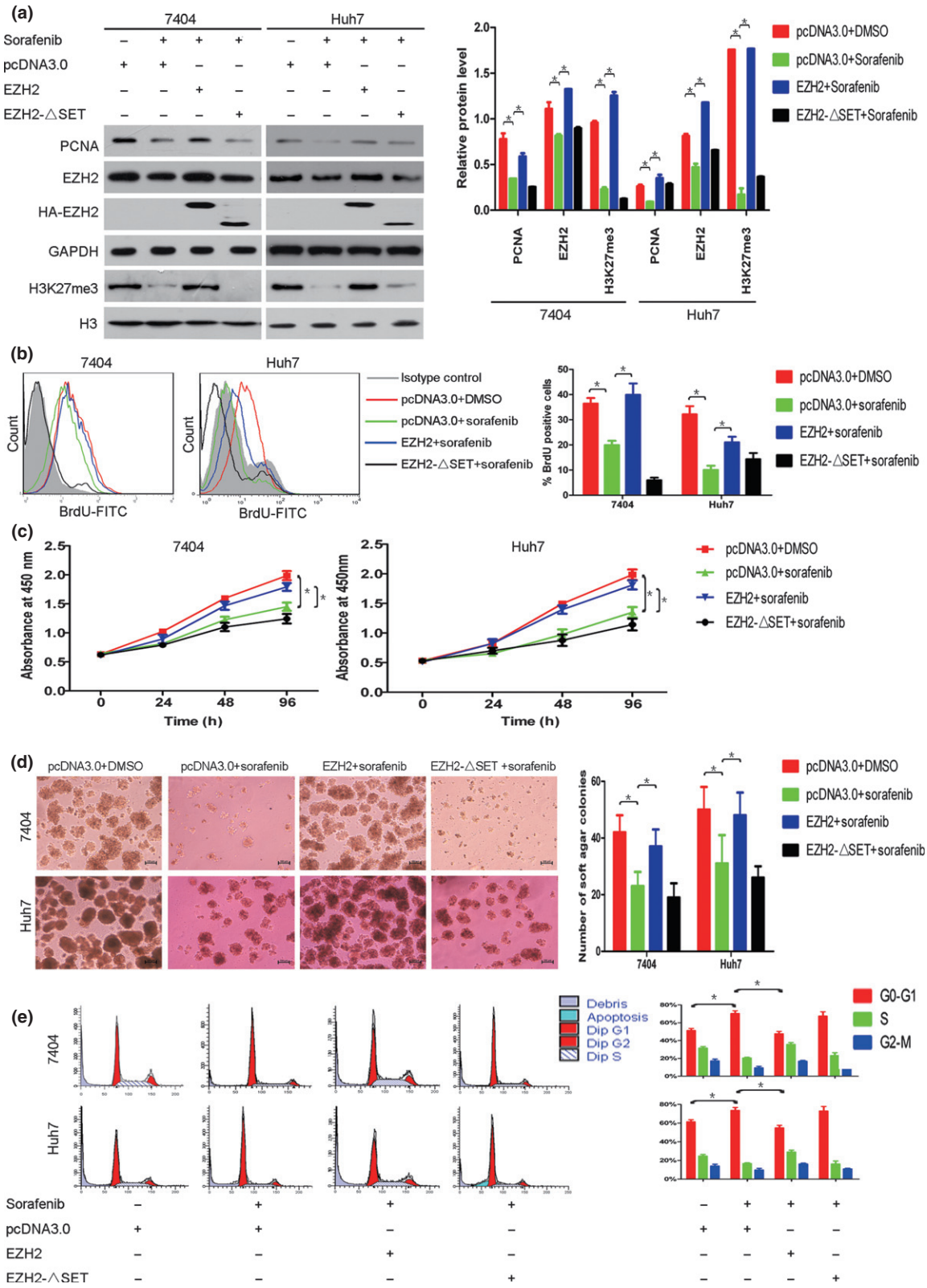

Fig. 3. Enhancer of zeste homolog 2 (EZH2) overexpression blocks sorafenib-induced hepatoma cell growth inhibition dependent on histone methyltransferase activity. Hepatoma 7404 and Huh7 cells were stably transfected with control vector, wild-type EZH2 plasmid, and histone methyltransferase deletion mutant EZH2-ΔSET plasmid. (a) Western blot analysis of proliferating cell nuclear antigen (PCNA), EZH2, HA tag, GAPDH, trimethylation of lysine 27 of histone H3 (H3K27me3), and total H3 protein levels after sorafenib (5 μM) treatment for 48 h. (b) Flow cytometry analysis for BrdU incorporation after sorafenib (5 μM) treatment for 48 h. (c) Cell proliferation assay for cell growth analysis with sorafenib (5 μM) treatment for the indicated times. (d) Colony formation assay for anchorage-independent growth analysis with sorafenib (5 μM) treatment for 14 days. (e) Flow cytometry analysis for cell cycle status after sorafenib (5 μM) treatment for 24 h. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a *t*-test (*$P < 0.05$).

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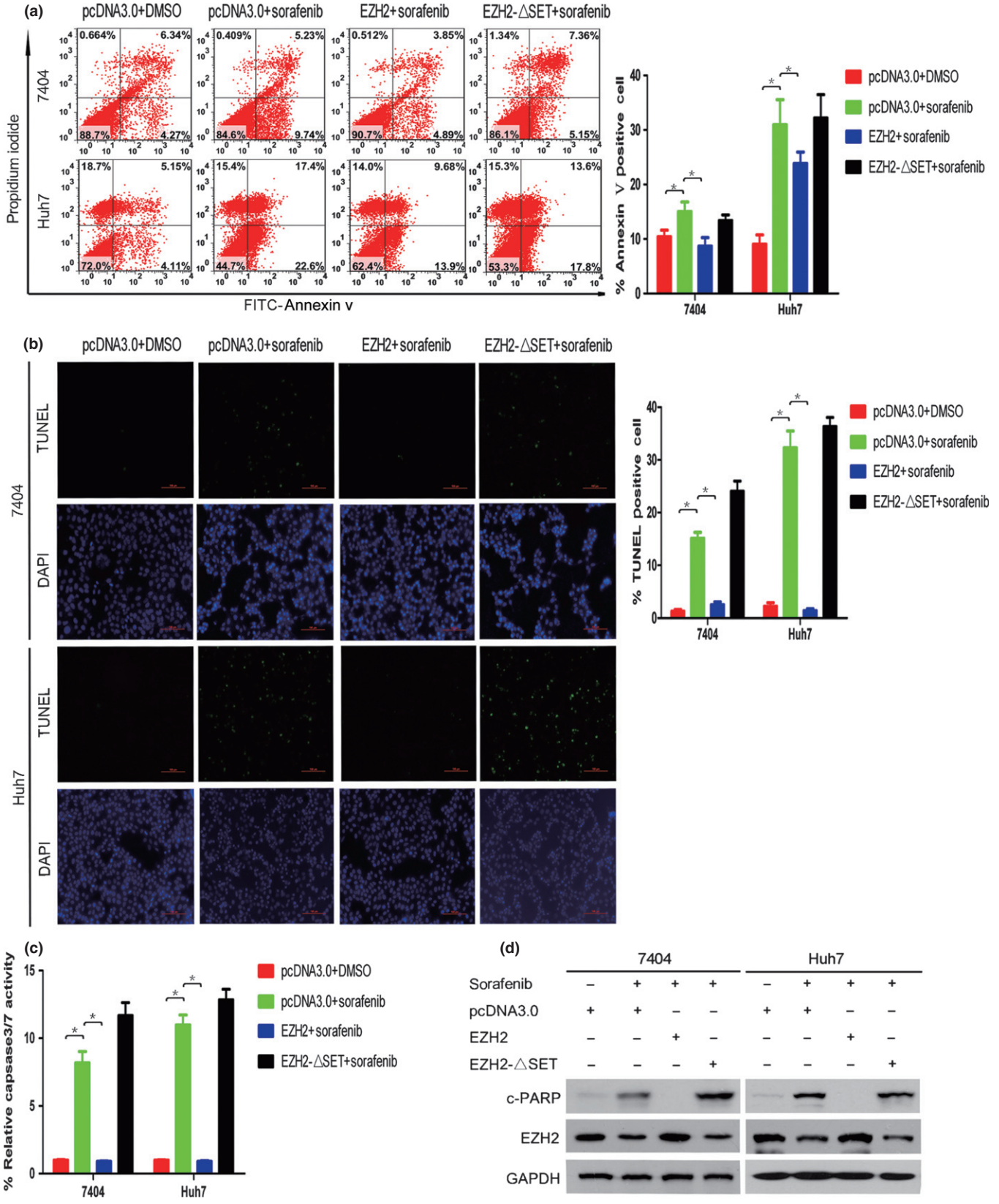

Fig. 4. Enhancer of zeste homolog 2 (EZH2) overexpression rescues sorafenib-induced hepatoma cell apoptosis dependent on histone methyltransferase activity. Flow cytometry analysis for Annexin V/propidium iodide staining (a), TUNEL assay (b), caspase 3/7 activity assay (c), and Western blot analysis of cleaved poly(ADP-ribose) polymerase (c-PARP), EZH2, and GAPDH (d) in 7404 and Huh7 cells stably transfected with control vector, wild-type EZH2 plasmid, and histone methyltransferase deletion mutant EZH2-ΔSET plasmid after sorafenib (5 μM) treatment for 48 h. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a *t*-test (*$P < 0.05$).

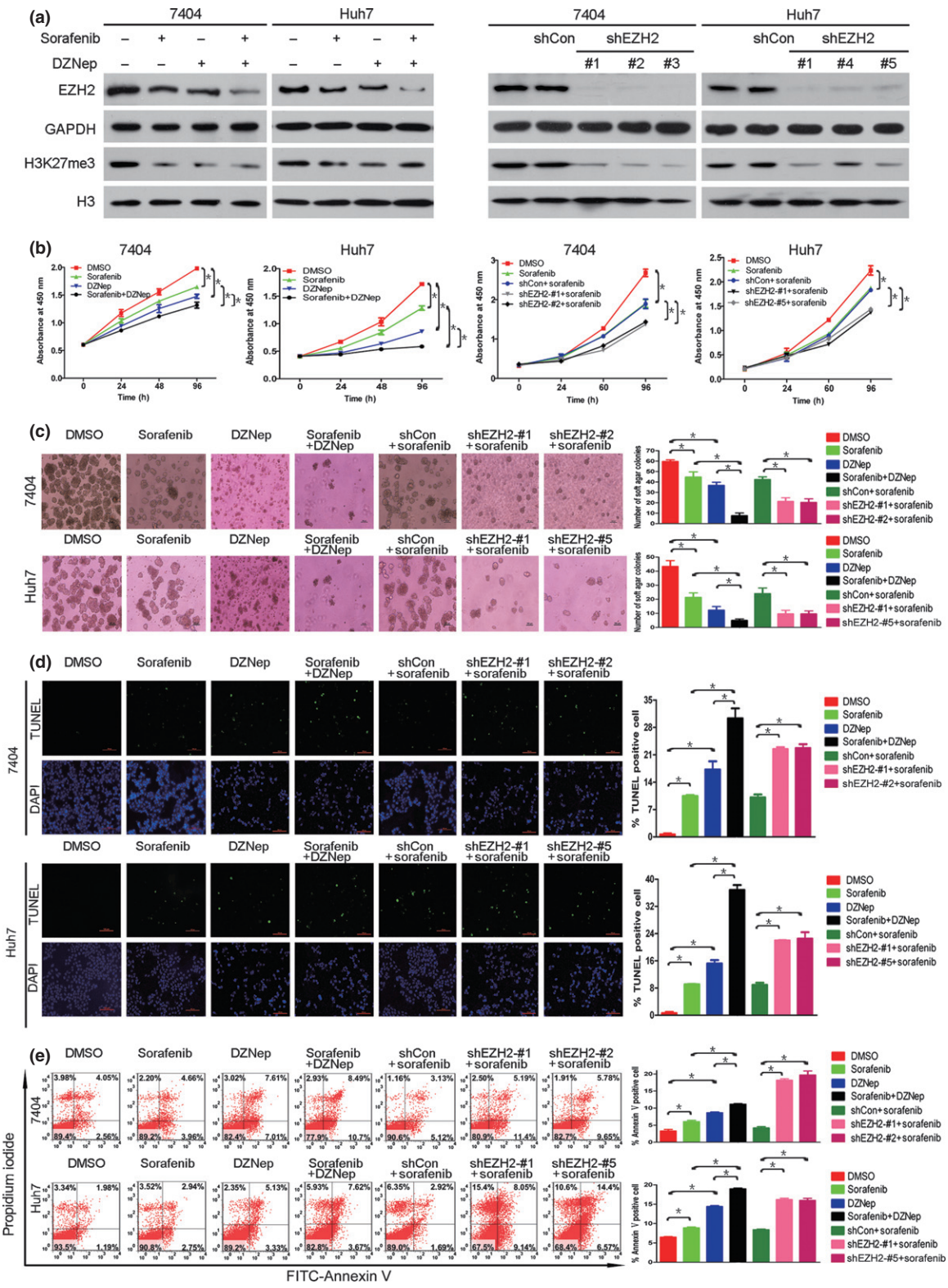

Fig. 5. Enhancer of zeste homolog 2 (EZH2) knockdown or inhibition promotes sorafenib-induced hepatoma cell growth arrest and apoptosis. (a) Western blot analysis of EZH2, GAPDH, trimethylation of lysine 27 of histone H3 (H3K27me3), and total H3 protein levels in 7404 and Huh7 cells after sorafenib (2.5 μM) treatment with or without 3-deazaneplanocin A (DZNep; 5 μM) for 48 h (left), and stably transfected with control shRNA (shCon) or specific EZH2 shRNA (shEZH2) (right). (b–e) Cell proliferation assay for cell growth analysis with sorafenib (2.5 μM) treatment for the indicated times (b), colony formation assay for anchorage-independent growth analysis with sorafenib (2.5 μM) treatment for 14 days (c), TUNEL assay after sorafenib (2.5 μM) treatment for 48 h (d), and flow cytometry analysis for Annexin V/propidium iodide staining after sorafenib (5 μM) treatment for 48 h (e) in 7404 and Huh7 cells with or without DZNep (5 μM) cotreatment, or stably transfected with control shRNA or specific EZH2 shRNA. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a *t*-test (**P* < 0.05).

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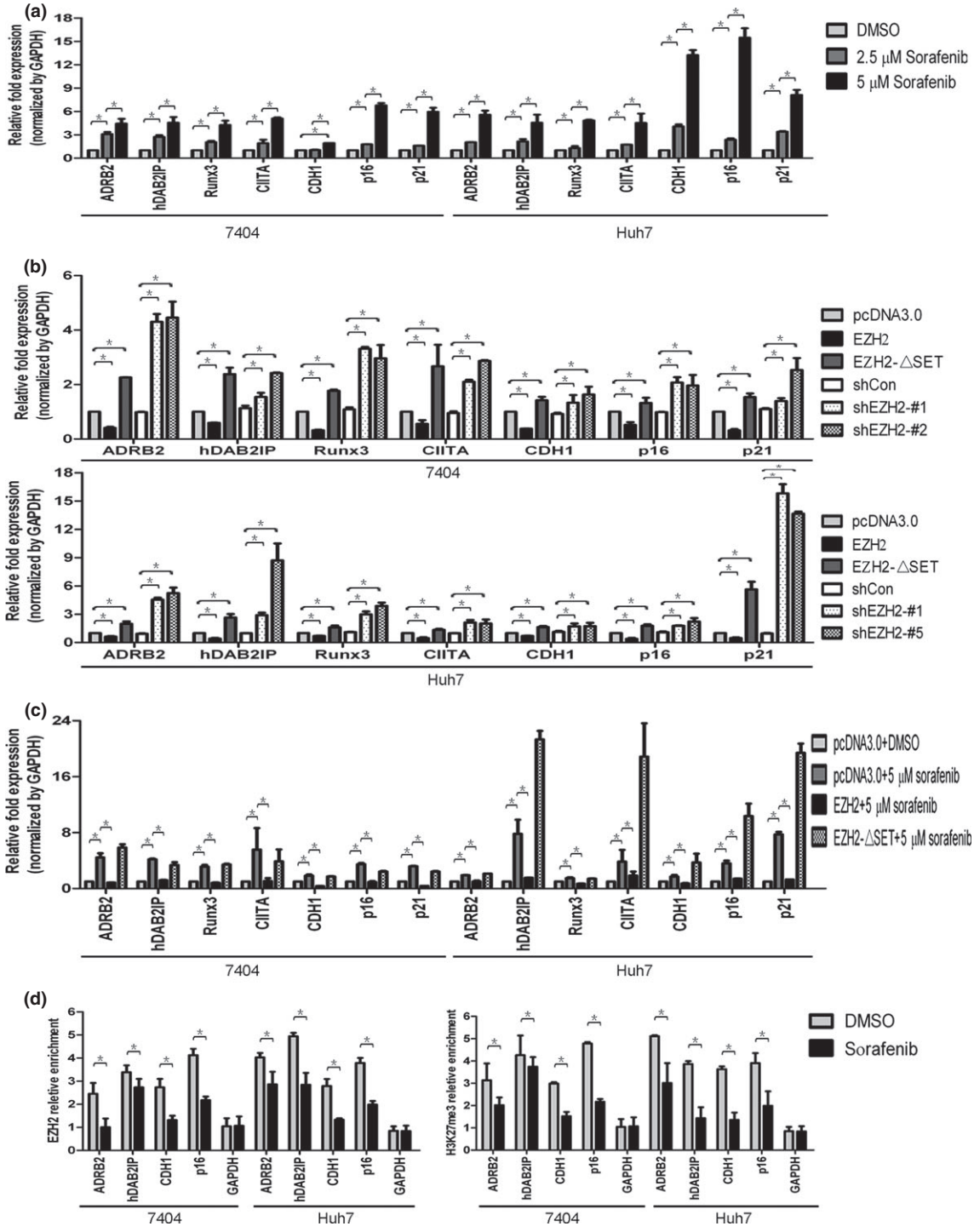

Fig. 6. Sorafenib upregulates transcription of polycomb repressive complex 2 (PRC2) target genes through enhancer of zeste homolog 2 (EZH2) declination in hepatoma cells. (a–c) Quantitative RT-PCR analysis for mRNA levels of PRC2 target genes including *ADRB2*, *DAB2IP*, *Runx3*, *CIITA*, *CDH1*, *p16*, *p21*, and control gene *GAPDH* in 7404 and Huh7 cells after sorafenib treatment for 48 h (a), 7404 and Huh7 cells stably transfected with control vector, wild-type EZH2 plasmid, histone methyltransferase deletion mutant EZH2-ΔSET plasmid, control shRNA (shCon), or specific EZH2 shRNA (shEZH2) (b), and 7404 and Huh7 cells stably transfected with control vector, wild-type EZH2 plasmid, and histone methyltransferase deletion mutant EZH2-SET plasmid after sorafenib (5 μM) treatment for 48 h (c). (d) ChIP assay for analyzing the recruitment of EZH2 (left) and the occupancy of the trimethylation of lysine 27 of histone H3 (H3K27me3) histone mark (right) at the promoters of PRC2 target genes including *ADRB2*, *DAB2IP*, *CDH1*, and *p16* and control gene *GAPDH* in 7404 and Huh7 cells after sorafenib (5 μM) treatment for 48 h. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a *t*-test (**P* < 0.05).

mechanism underlying sorafenib treatment for HCC, and implicate EZH2 inhibitor DZNep as a promising combination therapy to synergize sorafenib against advanced HCC.

Although sorafenib has been shown to have survival benefits in patients with advanced HCC in large clinical trials, increasing evidence has indicated two major challenges to its use:

only limited patients respond to treatment; and many rapidly acquire resistance. Regarding the molecular events responsible for acquired resistance of hepatoma cells to sorafenib, aberrant activations of signaling cascades such as PI3K/AKT have attracted much attention in recent years. Global epigenetic disturbance, which is thought to contribute to carcinogenesis through oncogene activation, loss of imprinting, genomic instability, and harmful expression of inserted viral sequences, might also be involved in acquired resistance of hepatoma cells to sorafenib and remains poorly understood. Our present study reveals the pivotal role of epigenetic modulator EZH2 in sorafenib-induced hepatoma cell growth arrest and apoptosis, implicating EZH2-mediated epigenetic modifications might be another crucial molecular mechanism underlying acquired resistance to sorafenib. Accumulated evidence indicates that some of the molecular targets of sorafenib can regulate EZH2 expression at transcriptional, post-transcriptional and post-translational levels.[35–37] A recent report indicates that CDK1-mediated EZH2 phosphorylation at Thr345/487 promotes EZH2 ubiquitination and subsequent degradation.[38] These molecular events might be responsible for sorafenib-mediated EZH2 degradation and merit further exploration. Other targets of sorafenib can enhance the effectiveness of EZH2 deletion in a synergistic manner.[39–42] Moreover, the epigenome is dynamic and therefore responsive to environmental signals not only during the critical periods in development but also later in life as well. Sorafenib, as an environmental factor, may alter epigenetic homeostasis by direct or indirect mechanisms, in which other epigenetic modifiers, besides EZH2, might also be involved.

A previous study revealed that EZH2-mediated concordant epigenetic repression of Wnt antagonists could contribute to constitutive activation of Wnt signaling and consequent proliferation of hepatoma cells.[21] In addition, EZH2 could also epigenetically silence multiple tumor suppressor miRNAs such as miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b to promote liver cancer metastasis.[22] Another study revealed that tumor suppressive microRNA-124 modulated the aggressiveness of HCC cells by repressing Rho-associated protein kinase 2 and EZH2.[20] High expression of oncogenic long non-coding RNA lncRNA-HEIH in HCC could facilitate tumor growth through directing EZH2 and repression of EZH2 target genes.[24] Interferon-α/signal transducer and activator of transcription 2 could suppress oncogenic p53-paralog DNp73 expression through the recruitment of the EZH2 polycomb group transcriptional repressor in liver cancer cells.[25] Our results also identified *ADRB2*, *DAB2IP*, *CDH1*, and *p16* as PRC2 target genes regulated by EZH2-mediated epigenetic alterations in a dose-dependent manner in hepatoma cells after sorafenib treatment. As *ADRB2*, *DAB2IP*, *CDH1*, and *p16* have been reported to be involved in EZH2-mediated tumorigenesis,[43–46] the potential functional significance of these EZH2-silenced genes awaits further investigation in hepatocarcinogenesis. Furthermore, EZH2 inhibits the promoter activities of target genes in a dose-dependent manner, which accounts for the synergistic effect of EZH2 inhibition and sorafenib treatment.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Whittaker S, Marais R, Zhu AX. The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* 2010; **29**: 4989–5005.
3 Llovet JM, Burroughs A, J B. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907–17.
4 Thomas MB, Abbruzzese JL. Opportunities for targeted therapies in hepatocellular carcinoma. *J Clin Oncol* 2005; **23**: 8093–108.
5 Llovet JM, Ricci S, Mazzaferro V *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.
6 Zhu AX, Duda DG, Sahani DV, Jain RK. HCC and angiogenesis: possible targets and future directions. *Nat Rev Clin Oncol* 2011; **8**: 292–301.
7 Villanueva A, Minguez B, Forner A, Reig M, Llovet JM. Hepatocellular carcinoma: novel molecular approaches for diagnosis, prognosis, and therapy. *Annu Rev Med* 2010; **61**: 317–28.
8 Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. *CA Cancer J Clin* 2009; **59**: 111–37.
9 Cao R, Wang L, Wang H *et al.* Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 2002; **298**: 1039–43.
10 Cao R, Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr Opin Genet Dev* 2004; **14**: 155–64.
11 Suva ML, Riggi N, Janiszewska M *et al.* EZH2 is essential for glioblastoma cancer stem cell maintenance. *Cancer Res* 2009; **69**: 9211–8.
12 Sudo T, Utsunomiya T, Mimori K *et al.* Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. *Br J Cancer* 2005; **92**: 1754–8.
13 Weikert S, Christoph F, Kollermann J *et al.* Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. *Int J Mol Med* 2005; **16**: 349–53.
14 Bachmann IM, Halvorsen OJ, Collett K *et al.* EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *J Clin Oncol* 2006; **24**: 268–73.
15 Kleer CG, Cao Q, Varambally S *et al.* EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A* 2003; **100**: 11606–11.
16 Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J* 2003; **22**: 5323–35.
17 Varambally S, Dhanasekaran SM, Zhou M *et al.* The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 2002; **419**: 624–9.
18 Sasaki M, Ikeda H, Itatsu K *et al.* The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma. *Lab Invest* 2008; **88**: 873–82.
19 Chen Y, Lin MC, Wang H *et al.* Proteomic analysis of EZH2 downstream target proteins in hepatocellular carcinoma. *Proteomics* 2007; **7**: 3097–104.
20 Zheng F, Liao YJ, Cai MY *et al.* The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. *Gut* 2012; **61**: 278–89.
21 Cheng AS, Lau SS, Chen Y *et al.* EZH2-mediated concordant repression of Wnt antagonists promotes beta-catenin-dependent hepatocarcinogenesis. *Cancer Res* 2011; **71**: 4028–39.
22 Au SL, Wong CC, Lee JM *et al.* Enhancer of zeste homolog 2 (EZH2) epigenetically silences multiple tumor suppressor miRNAs to promote liver cancer metastasis. *Hepatology* 2012; **56**: 622–31.
23 Chen Y, Lin MC, Yao H *et al.* Lentivirus-mediated RNA interference targeting enhancer of zeste homolog 2 inhibits hepatocellular carcinoma growth through down-regulation of stathmin. *Hepatology* 2007; **46**: 200–8.
24 Yang F, Zhang L, Huo XS *et al.* Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor growth through enhancer of zeste homolog 2 in humans. *Hepatology* 2011; **54**: 1679–89.
25 Testoni B, Schinzari V, Guerrieri F, Gerbal-Chaloin S, Blandino G, Levrero M. p53-paralog DNp73 oncogene is repressed by IFNalpha/STAT2 through the recruitment of the Ezh2 polycomb group transcriptional repressor. *Oncogene* 2011; **30**: 2670–8.

doi: 10.1111/cas.12132

26 Chiba T, Suzuki E, Negishi M *et al.* 3-Deazaneplanocin A is a promising therapeutic agent for the eradication of tumor-initiating hepatocellular carcinoma cells. *Int J Cancer* 2012; **130**: 2557–67.
27 Tan J, Yang X, Zhuang L *et al.* Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev* 2007; **21**: 1050–63.
28 Liu H, Xu J, Zhou L *et al.* Hepatitis B virus large surface antigen promotes liver carcinogenesis by activating the Src/PI3K/Akt pathway. *Cancer Res* 2011; **71**: 7547–57.
29 Tang X, Milyavsky M, Shats I, Erez N, Goldfinger N, Rotter V. Activated p53 suppresses the histone methyltransferase EZH2 gene. *Oncogene* 2004; **23**: 5759–69.
30 Xu J, Yun X, Jiang J *et al.* Hepatitis B virus X protein blunts senescence-like growth arrest of human hepatocellular carcinoma by reducing Notch1 cleavage. *Hepatology* 2010; **52**: 142–54.
31 Xu J, Liu H, Chen L *et al.* Hepatitis B virus X protein confers resistance of hepatoma cells to anoikis by up-regulating and activating p21-activated kinase 1. *Gastroenterology* 2012; **143**: 199–212.
32 Zhang W, Xu W, Xiong S. Macrophage differentiation and polarization via phosphatidylinositol 3-kinase/Akt-ERK signaling pathway conferred by serum amyloid P component. *J Immunol* 2011; **187**: 1764–77.
33 Zhang W, Xu W, Xiong S. Blockade of Notch1 signaling alleviates murine lupus via blunting macrophage activation and M2b polarization. *J Immunol* 2010; **184**: 6465–78.
34 Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* 2008; **48**: 1312–27.
35 Fujii S, Tokita K, Wada N *et al.* MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes. *Oncogene* 2011; **30**: 4118–28.
36 Chen H, Gu X, Liu Y *et al.* PDGF signalling controls age-dependent proliferation in pancreatic beta-cells. *Nature* 2011; **478**: 349–55.
37 Lu C, Han HD, Mangala LS *et al.* Regulation of tumor angiogenesis by EZH2. *Cancer Cell* 2011; **18**: 185–97.
38 Wu SC, Zhang Y. Cyclin-dependent kinase 1 (CDK1)-mediated phosphorylation of enhancer of zeste 2 (Ezh2) regulates its stability. *J Biol Chem* 2011; **286**: 28511–9.
39 Meng XW, Lee SH, Dai H *et al.* Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. *J Biol Chem* 2007; **282**: 29831–46.
40 Chen YL, Lv J, Ye XL *et al.* Sorafenib inhibits transforming growth factor beta1-mediated epithelial-mesenchymal transition and apoptosis in mouse hepatocytes. *Hepatology* 2011; **53**: 1708–18.
41 Chen KF, Tai WT, Liu TH *et al.* Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3. *Clin Cancer Res* 2010; **16**: 5189–99.
42 Coriat R, Nicco C, Chereau C *et al.* Sorafenib-Induced Hepatocellular Carcinoma Cell Death Depends on Reactive Oxygen Species Production in vitro and in vivo. *Mol Cancer Ther* 2012; **11**: 2284–93.
43 Yu J, Cao Q, Mehra R *et al.* Integrative genomics analysis reveals silencing of beta-adrenergic signaling by polycomb in prostate cancer. *Cancer Cell* 2007; **12**: 419–31.
44 Min J, Zaslavsky A, Fedele G *et al.* An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. *Nat Med* 2010; **16**: 286–94.
45 Herranz N, Pasini D, Diaz VM *et al.* Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol Cell Biol* 2008; **28**: 4772–81.
46 Kotake Y, Cao R, Viatour P *et al.* pRB family proteins are required for H3K27 trimethylation and Polycomb repression complexes binding to and silencing p16INK4alpha tumor suppressor gene. *Genes Dev* 2007; **21**: 49–54.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary Materials and Methods.

Table S1. List of PCR primer sets used in the construction of plasmids.

Table S2. List of PCR primer sets used in the quantitative RT-PCR analysis.

Table S3. List of PCR primer sets used in the ChIP–quantitative PCR analysis.